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**14. ABSTRACT**

My work this year was in two parts. I completed a study started by others in the laboratory, on the DNA repair gene GADD45, that is induced by the Egr1 transcription factor (TF), as well as other TFs. Egr1 transcriptionally induced GADD45 expression as shown by an GADD45-promoter luciferase reporter construct combined with the use of mutated Egr1 binding sites in the promoter. This work was submitted for publication and is now in review. The second project was to find the target genes regulated by Egr1 in a high-throughput array manner by using our own, home-made promoter array, made in collaboration with two other laboratories in a neighboring Institute. Prostate cells (normal and cancer-derived) were subjected to stresses such as radiation and chemotherapy drugs. Egr1 is rapidly induced and this is turn binds to many promoters of genes that respond to the stresses. Using chromatin immunoprecipitation I capture the activated promoters and identify them by hybridization to the promoter array. The combined procedures has been called "ChIP on a chip". The genes discovered are validated by several protocols. This work is on-going. We hope to identify genes that may be useful in disease risk assessment, or by finding stage markers for diagnostic purposes.

**15. SUBJECT TERMS**
radiation,drugs, stress response, target genes

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**RESULTS (in reference to the Statement of work)**

**Task 1**. To test prostate cancer cell lines with nonfunctional p53 for their biological responses to ionizing radiation (IR)(Months 0-4). a, b. DU145 and several prostate cancer cells that I tested, responded only slightly to ionizing radiation to give a very low induction of Egr1. So, I focused on induction of Egr1 by UV irradiation treatment. Also, I tested chemotherapeutic drugs to induce Egr1 in these prostate cancer cells. We decided to use Doxorubicin, which is currently used as a chemotherapy drug for various cancers including prostate cancer. Doxorubicin (also called Adriamycin) has been shown to intercalate between the bases in double stranded DNA, poison topoisomerase II, generate free radicals, and possibly disrupt the functioning of the cell membrane. Doxorubicin induced Egr1 in DU145, M12 prostate cancer cells and 267B1 normal prostate cells, as early as 2 h after treatment and sustained induction was seen till the cells underwent apoptosis (~48h). For our further experiments we have chosen the doses of 25nm and 100nm after 5 h of induction. There was no induction of growth.

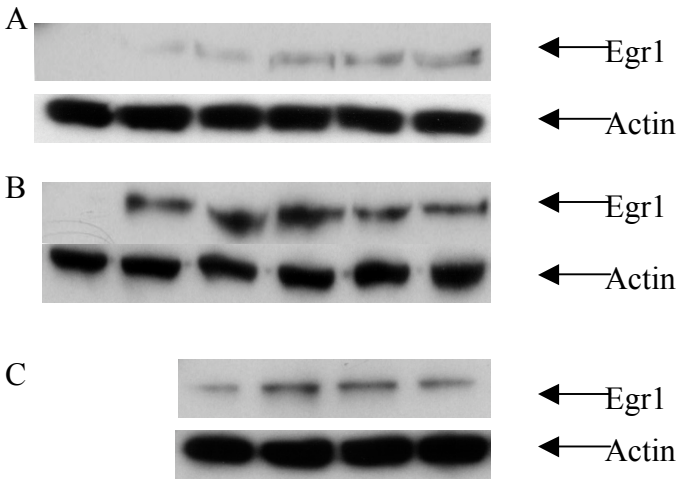


Figure 1: Egr1 induction on Doxorubicin treatment in DU145 cells. A. 2 h after induction with different doses of Doxorubicin (Control, 10nm, 25nm, 100nm, 1mm, 2mm), B. 5 h after induction, C. 48h after induction (Control, 10nm, 25nm, and 50nm), and cells treated with higher doses died by 48 h.

**Task1, c. and Task 2a and b**. To determine whether Egr1 plays an important role in such biological responses. (Months 5-12)
The proliferation of mouse embryo fibroblasts (MEFs) that were derived from Egr1-/ - and Egr1+/+ mouse embryos was tested before and after Doxorubicin treatment, with the result that proliferation was inhibited in proportion to the dose used (Fig. 2). This shows that Egr1 is required for the growth inhibitory response of Egr1 to chemotherapy drugs.

**Task 2.** We performed this task in a different way than originally planned because colleague Dr Jianxiu Yu in the lab showed that when DU145, M12 and other cell types are treated with expression vectors for Egr1 or some of its target genes (p53 and p73) apoptosis occurs to different degrees (J.Yu., T. Mustelin, and ED Adamson, submitted MS). He also showed that Etoposide (chemotherapy drug) induces Egr1 and interfering RNA to Egr1 prevents apoptosis, showing that Egr1 and its target genes all contribute to apoptosis.

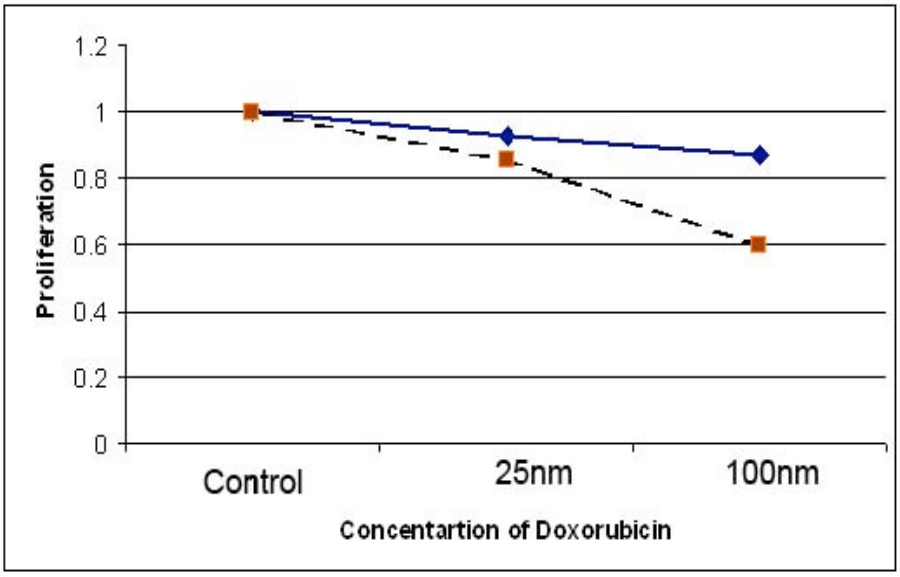


Figure 2Egr-/-MEFs( ——— ) andEgr+/+MEFs ( - - - - ) treated with Doxorubicin and cell proliferation was assessed after 24 hrs using WST-1 reagent (Roche Applied Sciences, IN) showing that cells containing Egr1 are more sensitive to Doxorubicin treatment and apoptosis.

**Task 3.** To identify Egr1 target genes upon irradiation or chemotherapy drug treatment, using ChIP on a chip.

When we started this study, I was using the promoter arrays with ~3000 promoter sequences on them but during the course of this one year we have constructed a newer version of the promoter array with more than 10,000 promoter sequences on them. The primer sets used for PCR were a kind gift from Dr. Michael J. Birrer (Chief Molecular Mechanism Section, NCI). The promoter array consists of 2 slides and the entire gene set has been spotted in triplicates (Figure 3). I worked closely with the groups of Dr. Michael McClelland and Dr. Dan Mercola (Sidney Kimmel Cancer Center, La Jolla) to construct these promoter arrays.

I have been trying various different stimuli to identify newer targets of Egr1 using ChIP on Chip under different kinds of stress for example UV, serum starvation and doxorubicin.

Some examples of the promoters that were bound by Egr1 under various stress stimuli were:

***UV stimulus***

p300 (co-activator, a known Egr1 target gene), PP1 (protein phosphatase 1), HSP70 (heat shock protein 70), H1 & H3d (histones), caveolin 1, deoxyribonuclease I-like 2, Cortactin

***Doxorubicin***

Oncostatin M, HSP40, vitronectin, TNFSF15, Cyclin E binding protein1, Bcl6 co-repressor, IL6 receptor, MAPKKK7, Inositol polyphosphate 5-phosphatase, Ribonucleoprotein A1, SUMO1 activating enzyme, subunit 1.

Clearly the most prominent target genes differ according to the stimulus. This is expected because each stimulus causes the regulation of a different set of signaling pathways that lead to slightly different biological endpoints.

I am in the process of analyzing the data and confirming this CHIP on Chip data with conventional CHIP procedure. Also, I am trying various other stimuli such as Etoposide and cisplatin treatment to see whether the Egr1 target genes change with different stresses. Recently, another postdoctoral fellow in the lab showed that there are two peaks in the expression of Egr1 on treatment with Etoposide (at 2 and 8h post treatment, Yu *et al*, unpublished data), therefore, we want to see the differences in target genes using CHIP on Chip analysis. We will next work very hard on determining a group of interesting stage marker genes or (more likely) the gene set that most strongly leads to apoptosis. This will require testing different stimuli and also combinations of treatments since the newest treatments are based on dual therapy regimens.

**Key Research Accomplishments**

1. Preparation of a paper for publication - Project on GADD45
When I arrived at the Adamson lab in July (2004), I continued work started by others but unfinished because there were missing data. The work included validation of GADD45 as a target gene of Egr1. I now have good western blots for GADD45 that could not be detected with immunoblotting alone. I used immunoprecipitation with anti GADD45a and analyzed this product for GADD45 on a western blot. I was able to write this data up and present it here. I am presenting a manuscript that we sent to Cell Death and Differentiation as a Letter to the Editor. They have reviewed it and made some suggestions for its resubmission before acceptance. (This is included in the Appendix).
2. Evaluation of the stress stimuli that cause the elevation of Egr1 expression in several prostate cancer cell lines. Evaluation of the physiological effects of radiation and doxorubicin treatment of several prostate cell-lines
3. Starting to validate the identification of Egr1 target genes after stress stimuli. Using the promoter array to reform the technique of ChIP on chip.

**Reportable Outcomes**
It is too early to report promoter array studies, but the submission and revision of a manuscript on GADD45 as a stress-responsive gene that is induced by Egr1 is in progress.

**Conclusions**
The work on the target genes of Egr1 after stress appears to be of very large scope. Individual target genes that have been discovered over the years from 1987 when the first description of Egr1 was issued. Now we have the means to discover the large-scale identification of Egr1 target genes and I am very excited to be a part of this work and thank you for your support.

**References**

1. Carrier, F., et al., *Characterization of human Gadd45, a p53-regulated protein.* J Biol Chem, 1994. **269**(51): p. 32672-7.
2. Virolle, T., et al., *The Egr-1 transcription factor directly activates PTEN during irradiation-induced signalling.* Nat Cell Biol, 2001. **3**(12): p. 1124-8.

3. Zhao, H., et al., *Activation of the transcription factor Oct-1 in response to DNA damage.* Cancer Res, 2000. **60**(22): p. 6276-80.
4. Hu, Z., S. Jin, and K.W. Scotto, *Transcriptional activation of the MDR1 gene by UV irradiation. Role of NF-Y and Sp1.* J Biol Chem, 2000. **275**(4): p. 2979-85.
5. Tran, H., et al., *DNA repair pathway stimulated by the forkhead transcription factor FOXO3a through the Gadd45 protein.* Science., 2002. **296**(5567): p. 530-4.
6. Huang, R.-P. and E.D. Adamson, *Characterization of the DNA-binding activity of the early growth response-1 (EGR-1) transcription factor: evidence for modulation by a redox mechanism.* DNA and Cell Biology, 1993. **12**: p. 265-273.
7. Yu, J., et al., *Co-activating Factors p300 and CBP are transcriptionally cross-regulated by Egr1 in prostate cells, leading to divergent responses.* Mol. Cell., 2004. **15**(1): p. 83-94.

## APPENDIX

**GADD45 is rapidly transcriptionally up-regulated by Egr1 in response to DNA damage**

Dear Editor-

A majority of cell lines and tissues respond to DNA-damaging stimuli such as irradiation by growth arrest, DNA repair and eventually apoptosis of damaged cells. The GADD45 gene family members are involved in these functions, and induction by p53 is a major mechanism for the transcriptional up-regulation of GADD45. However, in the absence of wild-type (wt) p53, as in a majority of cancer cells, we suggest that Egr1 (immediate early transcription factor) plays a major role. Since Egr1 is rarely mutated, this factor becomes an important mediator of cancer treatment by irradiation and chemotherapy.

A number of other transcription factors (TFs) including p53, have been shown to up-regulate GADD45 genes [1], but since Egr1 is a stress-induced transcriptional regulator of a pathway leading to apoptosis, we hypothesized that GADD45α and β might be Egr1 targets. The promoters of both genes have putative Egr1 binding sites and here we have tested GADD45α. First, we performed immunoblotting of DU145 prostate cancer cells after exposing cells to increasing doses of UV-C to measure the levels of Egr1 and GADD45α. The result in Figure 1A shows that both proteins were induced maximally at a dose of $40 J/m^2$. We also did a time course of analysis after irradiation from 0 5 h and calculated the relative fold-change of the level of expression of both the genes. We show in Figure 1B that Egr1 is expressed maximally at 1h while GADD45α is highest at 2h, a finding that fits the hypothesis. We also tested other stimuli such as the tumor promoter TPA and etoposide, a chemotherapy drug, with similar results (data not shown).

Mouse embryo fibroblasts (MEFs) derived from Egr1 gene knockout animals, were tested and compared with wild type (wt) MEFs to indicate the importance of Egr1. The results shown in Figure 1C indicate that only the wt Egr1+/+ cells are susceptible to Egr1 and GADD45α induction after TPA or other DNA damaging stimuli (such as UV, data not shown). In these cells the time course of maximal mRNA expression was exactly as shown in Figure 1B confirming that Egr1 precedes GADD45α expression after UV irradiation. Since Egr1 and GADD45α proteins are induced after these stimuli, then we should be able to detect Egr1 protein binding to the promoter of GADD45α using chromatin immunoprecipitation to crosslink the protein to the promoter DNA. After reversing the crosslinks and recovering the DNA, a PCR analysis was made using primers

designed to span the Egr1 putative binding sites in the GADD45α promoter. The results in Figure 1D show that UV, IR and TPA all induce Egr1 which then binds to the GADD45α promoter in DU145 cells, as demonstrated by the bands of DNA from PCR shown in lanes 3. In contrast, untreated and serum treated cells donot show PCR amplified products indicating that Egr1 is not bound to the GADD45α promoter under these conditions. A negative control is shown for the promoter of the cyclophilin gene (CPH) which is not a target of Egr1.

The above suggests that GADD45α is a target of Egr1 when cells are induced by DNA damaging stimuli. Therefore, we also tested the GADD45α promoter as a luciferase reporter construct and whether it could be activated by transfection of Egr1 or by UV. The GADD45α promoter of 2.3Kb has two groups of Egr1 binding sites (Figure 1E) and we first made a deletion analysis of the promoter to determine where Egr1 responsive activity was located. Results indicated that the pGL3-0.6 kb was almost as responsive as the full-length promoter, indicating that the sites at –200 and at +200 might both be active (data not shown). A series of GADD45α promoter constructs were made with mutated sites shown as Mut1, Mut2, Mut3 and Mut4 in Figure 1E. These reporter genes were then transfected into HEK293T cells to measure their activities as inducers of GADD45α indicated as luciferase activity in Figure 1F. The results indicate HEK293T cells already are able to respond to the promoter (set 1 on the left) and this may be due to the inherent expression of Egr1 in these cells. In the middle set, exogenous Egr1 was transfected and this shows that the Mut3 double binding site was the most responsive to induction. UV stimulus gave a similar profile of activities with Mut4 now showing more clearly that the loss of the triple Egr1 binding site at –200 was not important. We conclude that the two sites in the 5'UTR at +200 are actively binding Egr1 to effect activation of the GADD45α promoter.

These results combined with published work, indicate that stress stimuli applied to a variety of cell types elicits apoptosis at a later time (24-48h) and indicates that Egr1 may play a role in the process of stress response, and that GADD45α is one of the genes induced, in a pathway that leads to DNA repair, among others leading to apoptosis. For example, Egr1-/- MEFs are resistant to UV and IR irradiation and survive this stimulus while the Egr1+/+ MEFs do not [2]. Because of the multiple activities of the GADD45α gene product in stress response that leads to growth checkpoint, cell cycle retardation, DNA repair responses and apoptosis, it is important that its induction is rapid. We suggest that only Egr1 can provide this kind of response among the several TFs that have been shown to take part in the regulation of the GADD45α gene. It has been reported that Oct1[3], NF-Y [4] and FOXO [5] can be induced by stresses, but this is indirect and likely to be slower than the 1h maximal induction time for Egr1 mRNA. In addition Egr1 like p53 can interact with and become activated by CBP and p300 or with APE/REF-1[6], thus strengthening the response to stress and suggesting that when p53 is mutated, Egr1 can make the appropriate responses in the activation of relevant target genes [7].

**Acknowledgements**

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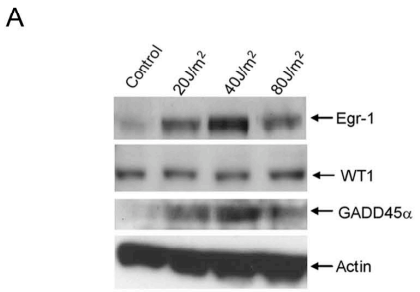
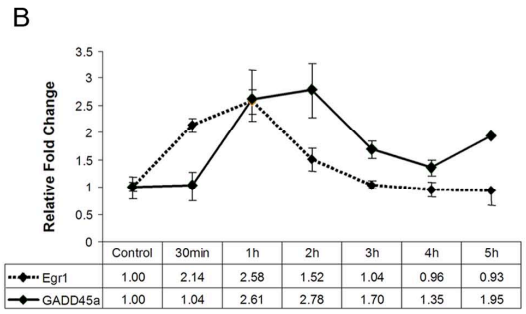
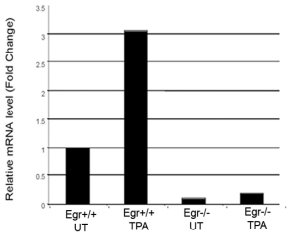
**Figure Legends**

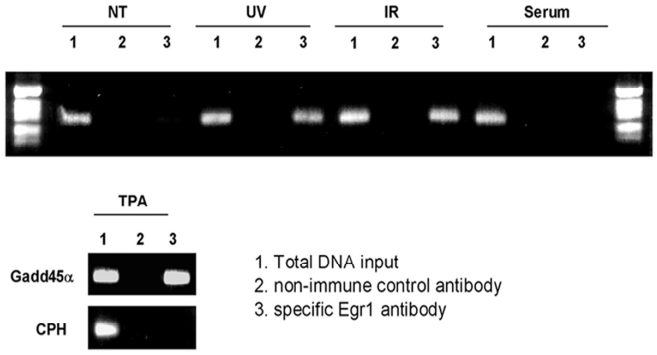
**Figure 1: A, Egr1 is induced earlier than GADD45α following UV irradiation or TPA treatment of prostate cancer DU145 cells**. To detect low levels of GADD45α, DU145 cell lysates were first immunoprecipitated using a rabbit antibody to GADD45α at a range of times after a 40 $Jm^{-2}$ stimulus. Sepharose A beads were added to the lysates to adsorb the antibody complex and the washed complexes were analyzed by immunoblotting. GADD45α was detected using a mouse anti-GADD45α antibody. **B**, a similar analysis to A after a range of radiation doses; **C**, Tumor promoter TPA induced Egr1 and GADD45α after 1-2 h of treatment: **C**, **Egr1 is required for GADD45α induction after UV-C irradiation.** Mouse embryo fibroblasts prepared from Egr1 null fibroblasts were unable to induce Egr1 or GADD45α. GADD45α mRNA levels measured by QRT-PCR in Egr1 null MEFs were little affected by TPA treatment, while Egr1 +/+ MEFs gave a three-fold induced level; **D**, **Egr1 binds to the GADD45α promoter after UV, IR or TPA induction.** Chromatin immunoprecipitation and PCR was used to demonstrate that Egr1 binds to the GADD45 promoter after these treatments of but not in untreated or a serum-treated cells., **E**, The structure of GADD45 reporter luciferase constructs is shown in studies to determine the effective Egr1 binding site. **F, The normalized transcriptional activity** of the GADD45α promoter constructs was measured 48h after transfection into HEK293T cells, of empty expression vector, or an Egr1 expression vector or after UV-C irradiation at 40 $J/m^2$. The proximal putative Egr1 binding sites were mutated as shown by the clear ovals in E. The relative luciferase signal was measured in Mut 1, Mut 2, Mut 3 (1+2), and Mut 4 reporter constructs in cells that were also transfected with empty vector (pcDNA) or with the Egr1 expression vector or with Egr1 induced by UV-C.

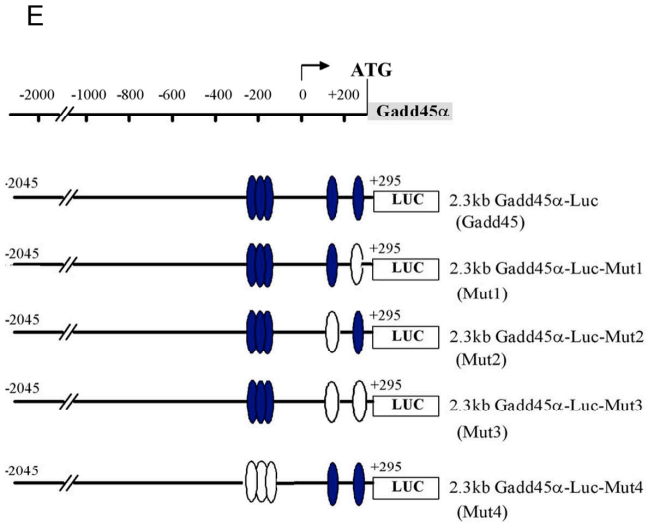


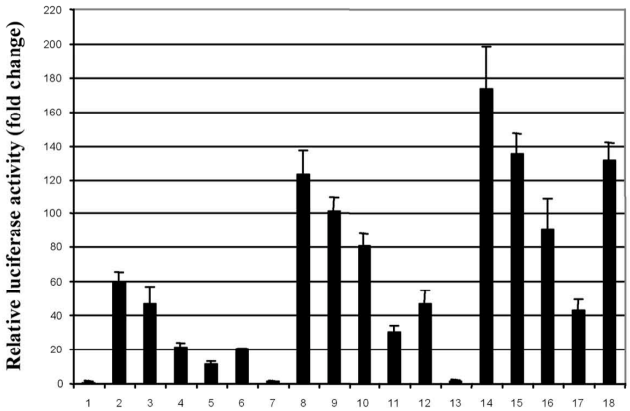




| | Control | 30min | 1h | 2h | 3h | 4h | 5h |
|---|---|---|---|---|---|---|---|
| Egr1 | 1.00 | 2.14 | 2.58 | 1.52 | 1.04 | 0.96 | 0.93 |
| GADD45a | 1.00 | 1.04 | 2.61 | 2.78 | 1.70 | 1.35 | 1.95 |




1 pGL3+pcDNA$_3$
2 Gadd+pcDNA$_3$
3 Mut1+ pcDNA$_3$
4 Mut2+ pcDNA$_3$
5 Mut3+ pcDNA$_3$
6 Mut4+ pcDNA$_3$
7 pGL3+Egr1
8 Gadd+Egr1
9 Mut1+ Egr1
10 Mut2+ Egr1
11 Mut3+ Egr1
12 Mut4+ Egr1
13 pGL3+pcDNA$_3$ (UV)
14 Gadd+pcDNA$_3$ (UV)
15 Mut1+ pcDNA$_3$ (UV)
16 Mut2+ pcDNA$_3$ (UV)
17 Mut3+ pcDNA$_3$ (UV)
18 Mut4+ pcDNA$_3$ (UV)

Figure 1
Arora et al